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Nuphar ×*fluminalis*, a New Hybrid from Central Japan

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The morphology, allozyme variation and pollen viability in nine populations of *Nuphar japonica, N. submersa*, and unidentified intermediate plants were examined. A phenogram based on cluster analysis using 10 morphological characters revealed three cluster groups. Groups 1 and 3 had distinctive morphological characteristics and corresponded to the published descriptions of *N. japonica* and *N. submersa*, respectively, whereas Group 2 showed intermediate values in most characteristics between the two species. In the allozyme study, many morphologically intermediate plants showed additive combinations of species-specific alleles in two loci (*lap1* and *mdh3*) from the two species. Pollen viability was significantly lower in intermediate plants than in the two species. Based on the evidence, we concluded that the intermediate plants were of hybrid origin between *N. japonica* and *N. submersa* and described them as *N.* ×*fluminalis.*

Key words: allozymes, hybridization, *Nuphar japonica*, *Nuphar submersa*, *Nuphar* ×*fluminalis*, pollen fertility

In the genus *Nuphar* Sm. (Nymphaeaceae), natural hybridization and introgression is well known and makes taxonomic delimitation of the species difficult (Heslop-Harrison 1953, Beal 1956, Padgett *et al.* 1998, 1999, 2002, Shiga and Kadono 2004, 2007). In Japan, the hybrid status of some plants intermediate between named species has been confirmed and the unstable conditions of the habitat have been discussed as playing an important role in hybridization (Shiga and Kadono 2007).

During a survey of *Nuphar submersa* Shiga and Kadono, a newly reported species from central Japan (Shiga *et al.* 2006), we found some unidentifiable plants. After morphological, allozymic and pollen viability studies, we concluded these plants to be hybrids between *N. japonica* DC., which is widely distributed in Japan (Kadono 1994), and *N. submersa*. In this paper we describe the new hybrid.

Materials and Methods

Plant materials

We collected 86 individual plants of *Nuphar* from 9 populations in central to eastern Japan (Table 1). The collections included specimens of *Nuphar japonica*, *N. submersa* and unidentified plants. Sampling occurred from August to early September in 2001, 2002, 2004 and 2005.

Morphological analyses

For measurements of morphological characteristics, we collected submerged leaves and flowers from 6 to 10 plants of *Nuphar* in each population (Table 1). We assessed the following 13 morphological characteristics that have been considered important in the identification of the taxa of *Nuphar* (Kadono 1994, Padgett *et al.* 1999, Shiga and

TABLE 1. Localities of 9 populations of *Nuphar* used in this study. Numbers of samples (N) for morphological measurements, allozyme analyses, and pollen viability are shown. All voucher specimens are deposited in OSA.

Pop. Code	Sampling locality	Latitude (N)	Longitude (E)	N	Voucher specimen
YA1	Yamagata Pref.: Tazawa, Murayama-shi	38°32'39"	140°22'12"	10	*T. Shiga 3120*
FS2	Fukushima Pref.: Sekisawa, Iitate-mura	37°40'49"	140°45'00"	10	*T. Shiga 3250*
NI4	Niigata Pref.: Nagamine, Yoshikawa-cho	37°14'44"	138°21'58"	10	*T. Shiga 3320*
IB2	Ibaragi Pref.: Tamasaki, Tamari-mura	36°09'32"	140°19'00"	10	*T. Shiga 3252*
TG1	Tochigi Pref.: Koshiro, Nikko-shi	36°39'	139°43'	10	*T. Shiga 3480*
TG2	Tochigi Pref.: Shimokawai, Nasukarasuyama-shi	36°42'	146°06'	10	*T. Shiga 3560*
TG3	Tochigi Pref.: Shimokomagi, Mooka-shi	36°26'10"	139°58'56"	6	*T. Shiga 3571*
TG4	Tochigi Pref.: Ohashi-cho, Sano-shi	36°19'00"	139°33'22"	10	*T. Shiga 3584*
TG5	Tochigi Pref.: Horigome-cho, Sano-shi	36°20'05"	139°34'05"	10	*T. Shiga 3587*

Kadono 2004, 2007, Shiga *et al.* 2006): length of submerged leaf blade (SL1), width of submerged leaf blade (SL2), shape of submerged leaf blade (SL3; SL1/SL2), sinus depth (SL4), sinus depth/length of blade ratio (SL5; SL4/SL1), maximum petiole width at 5 cm from the base of the blade (SL6), number of veins (SL7), maximum length of stigmatic disk (F1), stigma width (F2), number of stigmas (F3), anther length (F4), filament length (F5), ratio of anther length/filament length (F6; F4/F5). These measurement comprised 7 vegetative (SL1-SL7) and 6 floral (F1-F6) characteristics. All measurements were made using materials fixed in FAA (ethanol, formalin, and acetic acid) in the field.

The data for most quantitative characteristics were continuous, and it was difficult to recognize taxonomic units based on specific key characteristics. We therefore performed cluster analysis for the 9 populations based on the mean values of each characteristic. We chose Ward's method (Milligan 1980) for the analysis and used standardized variables and Euclidean distances.

Differences among populations and among cluster groups in each morphological trait were tested using nested analysis of variance (nested ANOVA). When the differences were significant among taxonomic groups ($P < 0.05$), we performed multiple comparison using Scheffé's F-test (Scheffé 1953). To analyze morphological relationships among individuals, we performed principal-component analysis (PCA). All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

To avoid misleading correlations, we excluded three characteristics (used for the calculation of ratios) from the cluster analysis and PCA: width of submerged leaf blade (SL2), sinus depth (SL4) and filament length (F5). As a result, 10 characters were used. Before applying the analyses, we arcsine-transformed the submerged leaf blade shape (SL3).

Allozyme analyses

We prepared extracts from 0.1 g of fresh leaves in 500 μL of grinding buffer (Soltis *et al.* 1983), and electrophoresed the extracts in 9% starch gels. The buffer systems, investigated enzymes and procedures of electrophoresis and staining were the same as in Shiga and Kadono (2007).

We detected 15 putative loci from the variation in banding patterns of the six enzymes. These loci were distributed as follows: a single locus for LAP, two loci each for PGI and PMI, three loci each for PGM and TPI, and four loci for MDH. Of these, 12 loci were genetically interpretable. All loci except *lap1* and *mdh3* could not be used for species-specific markers, so we determined the genotypes of *lap1* and *mdh3* and compared them with the different morphological groups.

Pollen viability

Pollen viability was estimated based on the

stainability of the pollen grains stained with a cotton blue staining solution (20% phenol, 20% lactic acid, and 40% glycerin aqueous solution to which 1% cotton blue aqueous solution was added) for 1 h. We removed three anthers from each flower and collected the pollen grains on a glass slide. We counted more than 500 pollen grains from each flower, and calculated pollen viability as the number of stained pollen grains divided by the total number of pollen grains examined.

In all analyses, we examined the same individuals (Table 1).

Results

Cluster groups

The Ward's phenogram revealed two major clusters (Fig. 1); the second cluster contained two distinct subclusters. We used these three clusters as operational taxonomic units and named them Groups 1, 2 and 3.

We found significant differences among the three cluster groups in all measured characteristics (nested ANOVA, $P < 0.05$; Table 2). Group 1 differed significantly from the other morphological group in 9 characteristics. It had the highest values in 9 characteristics (SL1, SL3, SL5, SL6, SL7, F1, F3, F4, F6). Group 3 differed significantly from the other morphological groups in 6 characteristics. Group 3 had the lowest values in 6 characteristics (SL1, SL5, SL7, F2, F4, F6). Group 2 differed significantly from the other groups in 5 characteristics, but had neither the highest nor lowest values in the measured characteristics. The values were thus intermediate between those in Groups 1 and 3 in 5 characteristics (SL1, SL3, SL7, F4, F6).

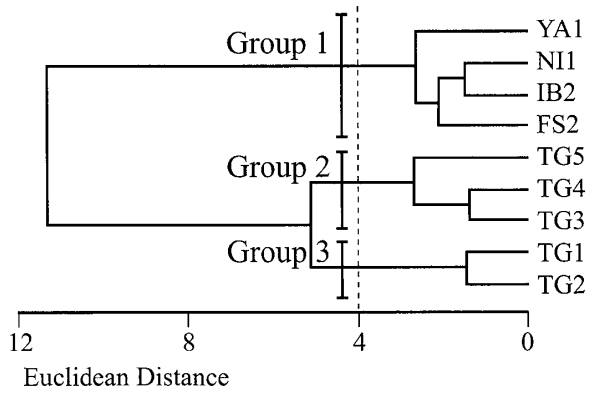

FIG. 1. Phenogram based on results of cluster analysis (Ward's method) of 9 populations of *Nuphar* in central to eastern Japan. Ten morphological characteristics were analyzed. Population codes are given in Table 1.

TABLE 2. Comparison of morphological characteristics of the cluster groups. Values within a row followed by letters differ significantly (Tukey's HSD multiple-comparison test, $P < 0.05$).

	Cluster group		
	1	2	3
Characteristic	Mean ± SD	Mean ± SD	Mean ± SD
Submerged leaf characteristics			
SL1 (cm)	32.3 ±5.8a	21.3 ±4.8b	13.1 ±3.1c
SL3 (SL2/SL1)	0.43±0.08a	0.33±0.06b	0.29±0.03b
SL5 (SL4/SL1)	0.19±0.04a	0.14±0.03b	0.04±0.04c
SL6 (mm)	5.5 ±1.4a	2.2 ±0.4b	1.6 ±0.4b
SL7 (no.)	64.8 ±6.1a	39.5 ±3.5b	29.1 ±5.5c
Flower characteristics			
F1 (mm)	10.6 ±2.3a	7.6 ±1.3b	6.4 ±0.4b
F2 (mm)	1.02±0.11a	0.86±0.07a	0.57±0.06b
F3 (no.)	14.3 ±2.6a	9.0 ±2.1b	7.5 ±0.9b
F4 (mm)	5.4 ±0.5a	4.0 ±0.6b	2.3 ±0.2c
F6 (Fl4/Fl5)	0.95±0.09a	0.68±0.14b	0.35±0.06c
Sample no. (n)	40	26	20

The plants in Groups 1 and 3 corresponded well to the descriptions of *Nuphar japonica* and *N. submersa*, respectively (De Candolle 1821, Shiga *et al.* 2006). Hereafter, we refer to Groups 1, 2, and 3 as *N. japonica*, intermediate plants, and *N. submersa*, respectively.

Morphological relationships among two species and intermediate plants

The PCA revealed well-expressed morphological relationships among the three morphological groups (Fig. 2a). The two species (Groups 1 and 3) and intermediate plants (Group 2) could be clearly distinguished along the PC axis 1 (Fig. 2a). The ranges of PCA values along axis 1 were 1.105 to

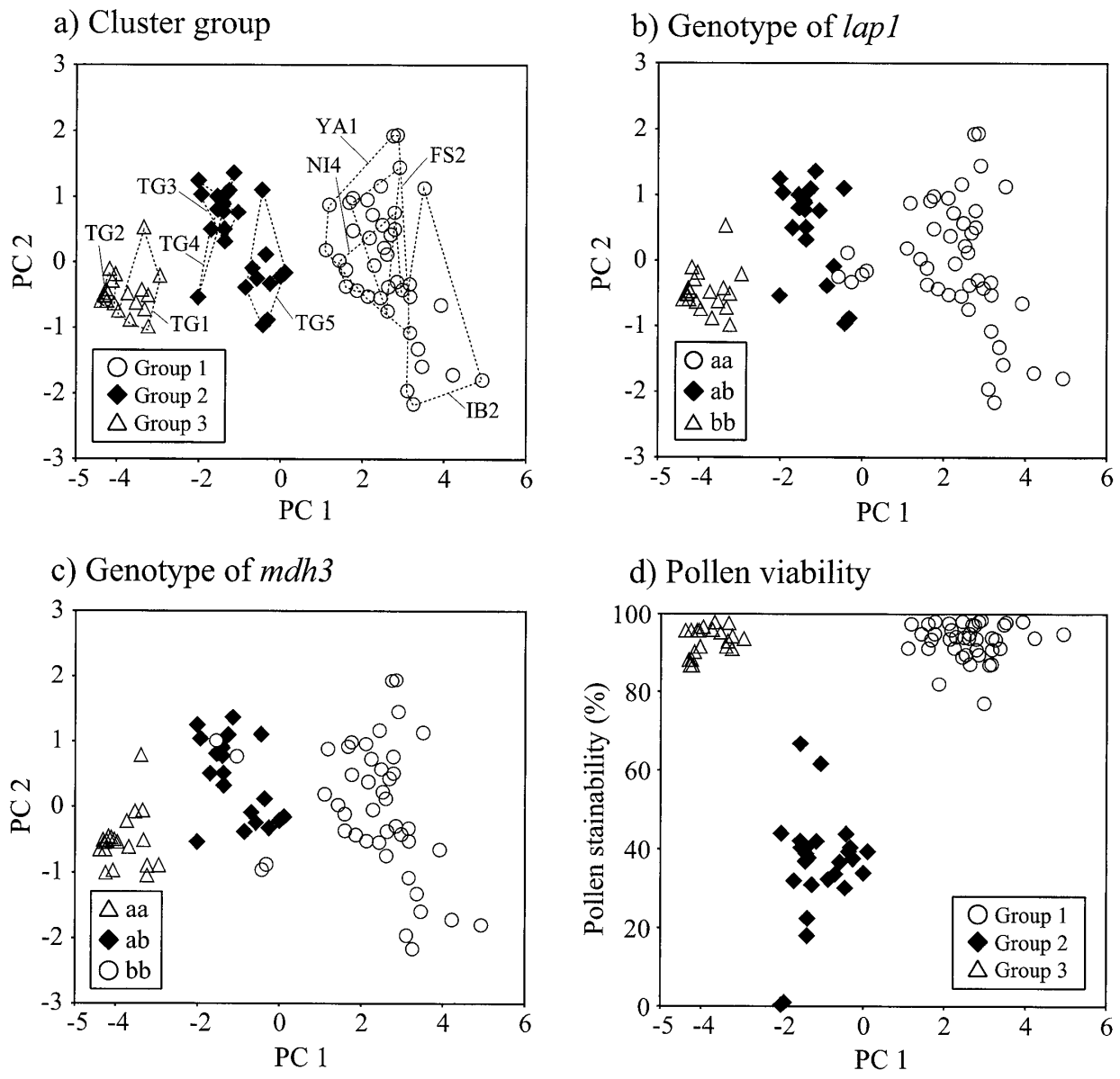

FIG. 2. Results of principal-components analysis (PCA) of 10 morphological characteristics in three cluster groups. (a) three cluster groups, (b) genotype of *lap1*, (c) genotype of *mhd3*, and (d) pollen viability superimposed on PCA (on PC axis 1 only). Symbols indicate phenotypes (a) and d)) or genotypes (b) and c)). In figure a), broken lines encircle same populations. For population code, see Table 1.

4.933 for *Nuphar japonica* (Group 1) and -4.397 to -2.951 for *N. submersa* (Group 3). Data for the intermediate plants (Group 2) were scattered between the ranges of values for *N. japonica* and *N. submersa* (-2.019 to 0.107). Morphological relationships were not well resolved in PC2 and PC3.

The first three principal components (PCs) accounted for 88.7% of the variance. PC1 explained 74.6% of total variance, and all characteristics except for SL3 contributed to this variance. PC2, which explained 7.4% of the total variance, was contributed to by none of the characteristics.

Genotype of lap1 *and* mdh3

Two polymorphic loci (*lap1* and *mdh3*) showed distinctive genotype frequencies among *Nuphar japonica*, *N. submersa*, and the intermediate plants (Figs. 2b, c, Table 3). The genotypes of *N. submer-*

sa and *N. japonica* were fixed as "aa" and "bb", respectively. In contrast, morphologically intermediate plants were not homozygous in the two loci, but most of the intermediate plants were "ab" heterozygous.

Pollen viability

Pollen viability was 93.2% ± 4.6% (mean ± SD) in *Nuphar japonica*, 93.1% ± 3.5% in *N. submersa*, and 35.5% ± 14.1% in the intermediate plants (Fig. 2d, Table 3). It was significantly lower in the intermediate plants than in either of the two species (Kruskal-Wallis test, $P < 0.001$).

In the intermediate plants, the pollen viability of the plants of heterozygous genotype in the two loci (30.6% ± 14.2%, n = 17) was lower than that of the plants, which is homozygous at one locus (44.4% ± 11.7%, n = 9) (Mann-Whitney's U test, $P = 0.031$).

Discussion

As can be seen from the phenetic relationships among the three cluster groups based on morphological characteristics, intermediate plants were scattered between *Nuphar japonica* and *N. submersa*. Secondly, the *lap1* and *mdh3* genotype of many morphologically intermediate plants was "ab" heterozygous, which is considered to be a reflection of crosses between "aa" and "bb" types (*N. japonica* and *N. submersa*). Third, intermediate plants showed low pollen stainability, although some intermediate plants exhibited moderate pollen stainability. The fruit set and seed set ratios of the intermediate populations were low in natural habitats (e.g. Figs. 3A-B). In addition, all intermediate populations were found within Tochigi Prefecture, where the two putative parental species occur sympatrically. *Nuphar japonica* is distributed in Japan and Korea (Lee 1989, Kadono 1994) and *N. submersa* is endemic to Tochigi Prefecture (Shiga *et al.* 2006). Our results indicate that the morphologically intermediate plants represent hybrids between *N. japonica* and *N. submersa.*

Some hybrid plants did not show additive combinations of the parental allozyme bands, as would be expected in F_1 hybrids, suggesting that sexual reproduction has occurred among the hybrids. The pollen viability of the putative later progeny was higher than in the putative F_1 hybrids. It is probable that backcrosses and/or crosses between hybrids have restored pollen viability in the hybrid *Nuphar* populations.

As hybrid backcrosses occur repeatedly, mor-

TABLE 3. Frequencies of multi-locus genotypes and mean pollen viability (%) for 9 populations of *Nuphar* from central to eastern Japan.

Population Code	Multi-locus genotype (*lap1* and *mdh3*) 1 (aa, bb)	2 (ab, bb)	3 (ab, ab)	4 (aa, ab)	5 (bb, aa)	Pollen viability (%) Mean	(SD)
***N. japonica* populations (Group 1)**							
YA1	1.000					94.1	(3.2)
FS2	1.000					93.1	(4.1)
IB2	1.000					90.9	(6.6)
NI4	1.000					94.6	(3.5)
Intermediate plants (Group 2)							
TG3		0.333	0.667			28.4	(29.2)
TG4			1.000			38.5	(4.3)
TG5		0.200	0.300	0.500		36.8	(4.2)
***N. submersa* populations (Group 3)**							
TG1					1.000	94.6	(2.4)
TG2					1.000	91.5	(3.9)

FIG. 3. *Nuphar* ×*fluminalis* Shiga & Kadono, hybr. nov. from TG4. A-B: Fruit. C: Holotype, *T. Shiga 3584* (OSA). Scale in A and B indicates 1 cm.

phological traits are believed to shift from intermediate values closer to the parental values (Arnold 1997, Lowe *et al.* 2004, Rosenthal *et al.* 2005) and pollen viability is assumed to be restored (Rieseberg and Noyes 1998). Our morphological study in three hybrid populations, however, indicated that morphological characters of the intermediate population strictly ranged between *Nuphar japonica* and *N. submersa*. Extensive introgression may not have occurred in this hybrid complex.

All of the hybrid populations were located in the irrigation ditches with seasonal water level fluctuation (T. Shiga and Y. Kadono, unpublished data). In *Nuphar* hybrids in Japan some populations of *N.*×*saijoensis* (Shimoda) Padgett and Shimoda and *N.*×*hokkaiensis* Shiga & Kadono occur in artificially fluctuating environments, such as in irrigation ponds (Shimoda 1991, Shiga and Kadono 2007). The hybrid between *N. japonica* and *N. submersa* may also be adapted to such a changing environment and speciation may be in progress.

Taxonomy

Nuphar ×**fluminalis** Shiga & Kadono, **hybr. nov.** (*N. japonica* × *N. submersa*) (Fig. 3)

Haec planta *Nuphari japonicae* DC. propinqua est, sed ab ea stigmatibus et antheris rufescentibus differt. Affinis *N. submersae* Shiga & Kadono, sed ab ea petiolis solidis et foliis sinuosis distant.

Typus. JAPAN, Tochigi Pref.: Sai-kawa River, Saikawa-bashi, Ohashi-cho, Sano-shi, Alt. 30 m, Aug. 7, 2005, *T. Shiga 3584* (holo- OSA; iso- TNS).

Intermediate between *N. japonica* and *N. submersa* in morphology. Submerged leaves narrowly ovate, base with a sinus (Fig. 3C). Filaments moderately recurved after anthesis. Stigmatic disc, pollen sack and fruit tinged with orange or red.

Japanese name. Nagare-kōhone (nov.)

Distribution. Japan. Endemic.

Habitat. Rivers and streams.

Other specimens examined. JAPAN, Tochigi Pref.: Uba-gawa River, Mizuhono-cho, Ashikaga-shi, May 2, 2003, *H. Kawauchi s.n.* (TOCH); Kurahone, Ohtawara-shi, *H. Kato s.n.* (TOCH 121885); Kuwa-mura, Oyama-shi, *K. Moritani 1359* (TOCH 12884); Kikusawa-gawa River, Funatsu-cho, Sano-shi, May 26, 2003, *H. Kawauchi s.n.* (TOCH); Kikusawa-gawa River, Horigome-cho, Sano-shi, *J. Hasegawa s.n.* (TOCH 137486), *T. Shiga 3587-3590, 3596, 4015-4017* (OSA); Ishida, Meiji-mura, Kawachi-gun, *K. Izawa s.n.* (TNS 70899); Egawa River, Shimokomagi, Mooka-shi, *T. Shiga 3568-3571, 4021* (OSA); Sai-kawa River, Ohashi-cho, Sano-shi, *T. Shiga 3578-3583, 4018-4020* (OSA).

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